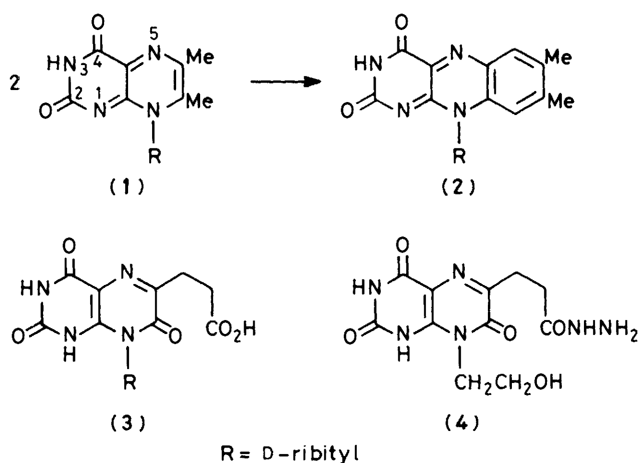
Specific Enzyme Inhibitors in Vitamin Biosynthesis. Part 5.[1] Purification of Riboflavin Synthase by Affinity Chromatography using 7-Oxolumazines†

Colin D. Ginger and Roger Wrigglesworth*
Departments of Biochemical Parasitology and Medicinal Chemistry, The Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS
W. David Inglis, Russel J. Kulick, Colin J. Suckling, and Hamish C. S. Wood*
Department of Pure and Applied Chemistry, University of Strathclyde, Glasgow G1 1XL

6-Carboxyethyl-7-oxo-8-D-ribityl-lumazine (**3**)† has been designed based on information about the mechanism of the enzymic reaction catalysed by riboflavin synthase (E.C. 2.5.1.9). This compound has been synthesised and used as a specific ligand for the affinity chromatography purification of the enzyme by attaching it in three different ways to Sepharose 4B. The effectiveness of each of these polymeric supports has been compared in order to define an optimised method for the purification of the enzyme.

Earlier papers in this series[2-5] have described the design and synthesis of specific inhibitors of riboflavin synthase (E.C. 2.5.1.9) which catalyses the conversion of two molecules of the lumazine (**1**) into riboflavin (**2**). It has been observed that the protons of the 7-methyl group of compound (**1**) undergo exchange for deuterium under chemical and enzymic conditions that lead to the formation of riboflavin.[4,5] A rationalisation of this result is that an anion is formed at the 7-methyl group through which both exchange and initiation of condensation occur. 7-Oxolumazines were proposed as transition-state (or reaction co-ordinate intermediate) analogues; a series of these compounds was synthesised and they were shown to be potent inhibitors of the enzyme.[2] The structure-activity profile of these compounds established an enzymic tolerance for a variety of substituents at the 6-position of the 7-oxolumazines. As a consequence of this, the 6-carboxyethyl-7-oxolumazine (**3**) was designed for use as an affinity chromatography ligand.[6] Further progress in the characterisation of the enzyme requires highly purified material, particularly with regard to affinity-labelling experiments designed to identify active-site residues involved in the catalytic process. The 7-oxolumazine (**3**) was synthesised using the standard procedure[2] and was shown to be a competitive inhibitor (K_i 1×10^{-5}M) of the enzyme from baker's yeast.



Results and Discussion

Preparation of Affinity Absorbents.—An affinity chromatography support was made by attaching the ligand (**3**) to Sepharose 4B. The latter was activated with cyanogen bromide and subsequently reacted with the 'spacer arm,' 4-azaheptane-1,7-diamine, using known procedures[7] to give an amino-functionalised polymeric derivative. The lumazine (**3**) was coupled to this polymer using the water-soluble condensing agent 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC.HCl).

The initial coupling reactions were not buffered and the pH of the reaction mixture after the reagents had been mixed was 4.6 and remained so throughout the coupling time. The immobilised product (A, Scheme) was isolated by filtration and extensive washing. This material showed a very different turbidometric u.v. spectrum [$\lambda_{max.}$ (pH 1) 270 nm] from that of free lumazine (**3**) ($\lambda_{max.}$ 329, 281 nm). The material A was very effective as an affinity chromatography ligand for the purification of riboflavin synthase (see below). The structure of this material and that of a product obtained from a related model reaction are the subject of the following paper.

In contrast, when the coupling reaction was carried out in a buffered medium at pH 6.7, the product had a bright blue fluorescence, characteristic of these 7-oxolumazines. This material B gave a u.v. spectrum [$\lambda_{max.}$ (pH 1) 329, 281 nm] identical with that of free lumazine (**3**), suggesting that the immobilised material has retained the 7-oxolumazine structure. The support B was also effective in the purification of the enzyme, but less so than A.

In order to have an independent check on the structures of these insolubilised materials, an alternative method of coupling the lumazine (**3**) to the Sepharose was devised which appeared to preclude the possibility of the formation of the immobilised product with the altered u.v. chromophore (*i.e.* material A). For reasons of synthetic convenience, this alternative coupling procedure was developed on the more accessible compound 8-(2-hydroxyethyl)lumazine. The hydrazide (**4**)[2] was coupled to carboxy-activated Sepharose 4B (commercially available CH-Sepharose) using the water-soluble carbodi-imide-mediated condensation process. Control experiments had established that there was no change in the u.v. chromophore of compound (**4**) on treatment with the carbodi-imide. The immobilised product showed a u.v. spectrum [$\lambda_{max.}$ (pH 1) 325, 280 nm] identical with that of (**4**).

Attention was then turned to the biologically relevant 8-D-

† In this paper, the name 'lumazine' is used to refer to the pteridine-2,4(1*H*,3*H*)-dione ring system.

ribityl series. The hydrazide (**5**) was made *via* the methyl ester from (**3**) and was coupled on to CH-Sepharose in an identical manner with that used for (**4**). This immobilised product C was effective in purifying riboflavin synthase. The oxolumazine (**3**) has also been attached to Sepharose using a triazine-mediated coupling method and the product was also effective in purifying riboflavin synthase.[8]

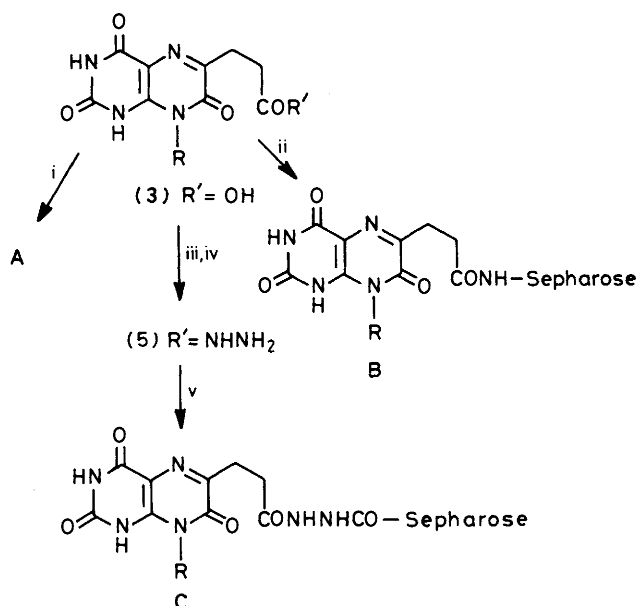
In order to quantify the amount of ligand attached to the Sepharose, a measured aliquot of the material was hydrolysed by refluxing in distilled water for 24 h. The u.v. spectrum of the filtered solution enabled the amount of coupled ligand to be calculated. Typical values ranged from 0.3—4 μmol/ml of settled gel.

Affinity Chromatography Experiments.—Before being applied to the column, the enzyme from baker's yeast was partially purified by a modification of the method of Plaut[9] in order to remove the particulate matter and some of the extraneous protein. This was necessary to maintain reasonable flow rates through the affinity column. Several different experiments have been carried out on each of these supports and the details given in the Experimental section are typical. Control columns of Sepharose 4B, AH-Sepharose (amino-functionalised), and CH-Sepharose (carboxy-functionalised) did not retain riboflavin synthase, although a slight retardation of the activity was observed on AH-Sepharose; this is presumably an ion-exchange effect. The recovery of the activity from these reference columns was essentially quantitative.

A typical elution profile using gel A is shown in the Figure. This shows that under these conditions, all of the riboflavin synthase activity was retained by the column whereas a large amount of extraneous protein ran straight through. Elution of the column with the enzymic substrate (**1**) (5×10^{-3}M) specifically removed all of the enzyme activity with a tight elution profile which is characteristic of efficient affinity chromatography.[10] When this same column was re-used under similar conditions, a small amount (<10%) of activity break-through was observed on loading, but quantitative recovery of the absorbed enzyme was achieved with substrate elution.

The determination of the degree of purification effected by the affinity column requires the measurement of the specific activity of the eluted enzyme. This has proved to be a difficult problem which has not been completely solved. Removal of the eluting substrate (**1**) from the highly purified enzyme by dialysis resulted in complete loss of the enzyme activity. Therefore, the specific activity in these early experiments was calculated indirectly by measuring the enzyme activity in the presence of the eluting substrate immediately after elution from the column. The protein concentration was measured on the inactive enzyme after dialysis. Purifications of enzyme of the order of 50-fold giving a final specific activity of 55—60 nmol riboflavin/min/mg protein have been achieved routinely with this column. Experiments using dialysis or protein precipitation were not successful and the best procedure was gel filtration on Sephadex G10. It is important to point out that there are uncertainties in the specific activity measurements on purified enzyme because of the difficulty of measuring accurately the very small amounts of purified protein. The attempted elution of the enzyme activity from affinity support A using a high ionic strength buffer (0.5M-KCl) or by altering the pH of the elution (0.1M-sodium acetate; pH 4.6) was unsuccessful.

Less work has been carried out on material B because it proved to be less effective. A significant break-through of activity was observed on loading and the activity profile obtained by eluting with the lumazine (**1**) (5×10^{-3}M) was very broad. The profile was improved when 10^{-2}M substrate was



Scheme. *Reagents:* i, amino-activated Sepharose, EDC.HCl, pH 4.6; ii, amino-activated Sepharose, EDC.HCl, pH 6.5; iii, H^+/MeOH; iv, NH_2NH_2–H_2O; v, carboxy-activated Sepharose, EDC.HCl.

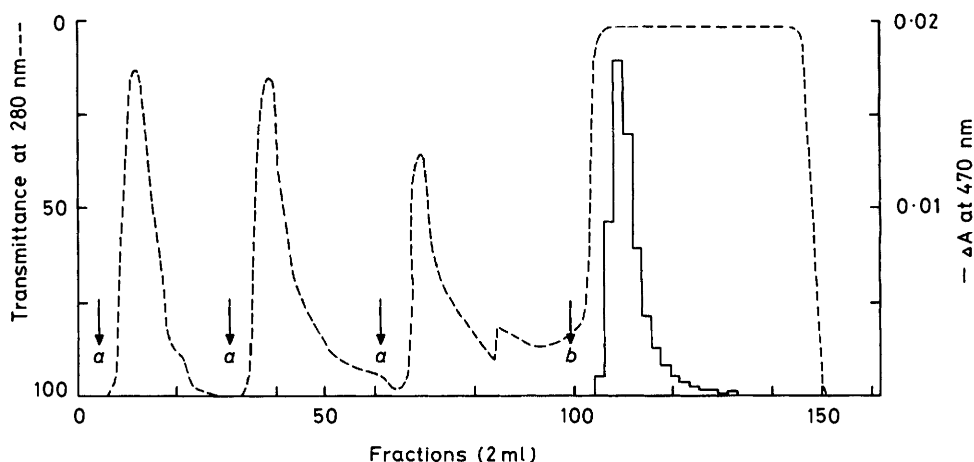


Figure. Affinity chromatography on material A. The crude enzyme was loaded at points *a* and elution with (**1**) was begun at *b*

used to elute the enzyme activity, but the recoveries were only 50—60%. Consequently, the purification factor of this material was not determined. We have not been able to repeat the very high purification reported in our preliminary communication.[6]

The capacity of material C to retain enzyme activity was very high with no break-through on loading. Attempts to saturate even small columns of this material have been unsuccessful, so a maximum capacity for binding activity could not be determined. A sharp elution of activity from the column again needed a high (10^{-2}M) substrate concentration, and recoveries of between 75—95% of the absorbed activity were routinely obtained.

Attempts to elute the enzyme with riboflavin, which is much cheaper and which is the product of the enzymic reaction, were not successful. It was discovered that, although no riboflavin synthase activity was eluted with riboflavin, a small amount of protein which had no activity was eluted. We attempted to detect other enzymic activities in this protein but negative results were obtained in assays for xanthine oxidase,[11] succinate dehydrogenase,[12] and peroxide-forming enzymes.[13]

The separation of the purified enzyme from the substrate has been investigated with material eluted from column C. Approximately 50% of the initial activity was recovered free from substrate under optimal conditions. The loss of eluted activity again made the calculation of the purification factors for the affinity column difficult to obtain. However, if the indirect calculation procedure is used, purifications of the order of 40—65 nmol riboflavin/min/mg protein have been achieved with support C.

The stability of the purified enzyme when separated from the substrate is very low, as previously reported by Plaut.[9] Storage at −80 °C resulted in 25% loss of activity in 3 days. A freeze-dried sample of (inactive) purified enzyme was examined by analytical electrophoresis on polyacrylamide gels. Although an initial run showed only one stained protein band, this could not subsequently be reproduced, and thus we have no absolute criterion of the purity of the enzyme. The specific activity of the material purified by affinity chromatography (40—65 nmol riboflavin/min/mg protein) is approximately the same as that of material purified by the long classical procedure (10 steps) developed by Plaut[14] (43 nmol riboflavin/min/mg protein).

In summary, the hydrazide-derived support C appears to be the material of choice for the purification of the enzyme. Elution using a high concentration of the expensive substrate (**1**) is the only effective method of removing activity specifically from the column, and the problem of removing the small-molecule co-eluant from the enzyme without loss of activity has been only partially resolved. The optimised operating conditions are described in the Experimental section.

Experimental

U.v. spectra were determined with Unicam SP 8000 A or Perkin-Elmer 402 spectrophotometers for aqueous solutions of standard pH. ^{1}H N.m.r. spectra were recorded using Perkin-Elmer R12 B (60 MHz), or Perkin-Elmer R14 (100 MHz) spectrometers operating in the continuous wave mode and Bruker HFX 90 or JEOL PS100: PFT100 instruments in the Fourier transform mode (tetramethylsilane as standard). Resonances are reported as p.p.m. from tetramethylsilane, position numbers indicating downfield shifts (*i.e.* δ scale). Ether refers to diethyl ether.

All the products were homogeneous when examined by paper chromatography (Whatman No. 1 paper) or by t.l.c. on silica gel plates, using the solvents previously described.[2] Spots were detected with filtered u.v. light (λ 254 and 365 nm).

Turbidometric u.v. spectra were run in the normal way, using a suspension of the appropriate gel which had just been vigorously shaken against a control of the same gel after settling.

The amount of Sepharose-bound ligand was estimated by suspending the gel (2 ml, settled volume) in water (25 ml) and refluxing for 24 h. Filtration to remove the residual insoluble material (u.v. transparent) and measurement of the absorbance at 348 nm of the filtrate allowed the calculation of the amount of coupled ligand (expressed as μmol/ml settled gel).

Coupling of 6-*Carboxyethyl*-7-*oxo*-8-D-*ribityl*-*lumazine* (**3**) *to Amino-functionalised Sepharose.*—(1) *At* pH 4.6. Sepharose 4B (25 g) (Pharmacia) was washed with water (3 × 20 ml) and then added to water (25 ml) in a beaker and stirred at room temperature. Cyanogen bromide (2.5 g) was added, the pH raised to 10—11, and kept there by addition of 2M-sodium hydroxide. Proton release continued for *ca.* 20 min. The mixture was filtered using suction and *rapidly* washed with ice-cold 0.1M-sodium hydrogen carbonate solution (pH 9) (300 ml). The damp suspension was transferred to further cold sodium hydrogen carbonate solution (40 ml) and aqueous 2M-4-azaheptane-1,7-diamine (20 ml) was added. The mixture was stirred for 5 h at 0 °C and then stored for 12 h at this temperature.

The mixture was filtered using suction and washed with 0.1M-$NaHCO_3$ (100 ml), H_2O (100 ml), 1M-HCl (100 ml), H_2O (100 ml), 1M-NaOH (100 ml), and H_2O (100 ml) as rapidly as possible.

The activated Sepharose was added to water (20 ml) and 6-carboxyethyl-7-oxo-8-D-ribityl-lumazine (**3**) (1 g) was added in water (20 ml). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.35 g) was added to effect the coupling. The pH was adjusted to 4.6 with sodium hydroxide and it subsequently remained constant throughout the reaction. The mixture was stirred for 3.5 h at room temperature by which time it had darkened considerably. Filtration of the inhibitor-coupled Sepharose was carried out under suction and the residue washed with 1M-K_2HPO_4 (100 ml), water (100 ml), KH_2PO_4 (100 ml), and water until the washings showed no absorbance in the u.v. The washed, coupled Sepharose was stored as an aqueous suspension at 4 °C until required for affinity chromatography. A turbidometric u.v. spectrum of this material showed $\lambda_{max.}$ (pH 1) 270 nm.

(2) *At* pH 6.7. The same procedure was followed except that the pH was adjusted from 4.6 to 6.7 by the addition of sodium hydroxide. A turbidometric u.v. spectrum of this material [$\lambda_{max.}$ (pH 1) 329, 281 nm] was directly superimposable on that of the lumazine (**3**).

Synthesis of 6-(2-*Hydrazinocarbonylethyl*)-8-(2-*hydroxyethyl*)*pteridine*-2,4,7(1H,3H,8H)-*trione* (**4**).—6-*Carboxyethyl*-8-(2-*hydroxyethyl*)*pteridine*-2,4,7(1H,3H,8H)*trione*. 6-(2-Hydroxyethylamino)-5-nitrosouracil[15] (2.5 g, 0.013 mol) was suspended in water (15 ml) and warmed on the steam-bath. Reduction of the nitroso group was achieved by the slow addition of solid sodium dithionate until the solution became pale yellow. The solution was filtered and the filtrate quickly adjusted to pH 2 using 2M-hydrochloric acid. The filtrate was immediately added to a solution of 2-oxoglutaric acid (2 g, 0.014 mol) in water (40 ml). The mixture was heated in the dark on the steam-bath under nitrogen for 4 h, left overnight at room temperature, and the volume reduced to 20 ml. The pale yellow crystals were filtered off and recrystallised (twice) from water to give the *pteridine* as off-white needles (2.6 g, 68%), m.p. 305—310 °C (decomp.) (Found: C, 44.7; H, 4.2; N, 18.6. $C_{11}H_{12}N_4O_6$ requires C, 44.6; H, 4.1; N, 18.9%); $\lambda_{max.}(\varepsilon)$ (pH 1) 281 (12 080) and 328 nm (12 700); $\lambda_{max.}$ (pH 13) 259 (7 380), 288 (9 140), and 352 nm (13 240); δ(D_2O–NaOD) 4.41 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 3.85 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 2.96 (2 H, m, 6-$CH_2CH_2CO_2H$), and 2.55 (2 H, m, 6-$CH_2CH_2CO_2H$); *m*/*z* 278 (32%), 260 (46), 234 (27), and 232 (100).

8-(2-Hydroxyethyl)-6-(2-methoxycarbonylethyl)pteridine-2,4,7(1H,3H,8H)-trione. The 6-carboxyethylpteridine (2.5 g, 8.4×10^{-3} mol) was suspended in dry methanol (200 ml) and 5 drops of concentrated sulphuric acid were added. The mixture was stirred under reflux for 4 h, and cooled slowly. The solution was refrigerated overnight, and the off-white crystals were collected. The product was recrystallised from methanol to give *8-(2-hydroxyethyl)-6-(2-methoxycarbonylethyl)pteridine-2,4,7-(1H,3H,8H)-trione* (1.7 g, 65%), m.p. 255—256 °C (Found: C, 46.5; H, 4.6; N, 18.0. $C_{12}H_{14}N_4O_6$ requires C, 46.4; H, 4.6; N, 18.1%); $\lambda_{max.}(\varepsilon)$ (pH 1) 283 (13 100) and 328 nm (12 900); $\lambda_{max.}$ (pH 13) 260 (9 300), 288 (10 700), and 353 nm (14 700); $\delta(D_2O-NaOD)$ 4.37 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 3.85 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 3.35 (3 H, s, CO_2CH_3), 2.93 (2 H, m, 6-$CH_2CH_2CO_2H$), and 2.53 (2 H, m, 6-$CH_2CH_2CO_2H$).

6-(2-Hydrazinocarbonylethyl)-8-(2-hydroxyethyl)pteridine-2,4,7-(1H,3H,8H)-trione (4). The 6-(2-methoxycarbonylethyl)-pteridine (1.0 g, 3.2×10^{-3} mol) was dissolved in hydrazine hydrate (20 ml) and stirred at room temperature for 30 min. The hydrazide product crystallised out spontaneously, or on addition of ethanol (75 ml). Recrystallisation from 2M-acetic acid gave *6-(2-hydrazinocarbonylethyl)-8-(2-hydroxyethyl)-pteridine-2,4,7(1H,3H,8H)-trione* (**4**) as pale yellow crystals (0.6 g, 60%), m.p. 240—241 °C (Found: C, 42.6; H, 4.6; N, 26.9. $C_{11}H_{14}N_6O_5$ requires C, 42.6; H, 4.6; N, 27.1%); $\lambda_{max.}(\varepsilon)$ (pH 1) 280 (13 500) and 328 nm (13 650); $\lambda_{max.}$ (pH 13) 261 (10 300), 288 (9 330), and 356 nm (14 500); $\delta(D_2O-NaOD)$ 4.39 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 3.84 (2 H, t, *J* 6 Hz, 8-CH_2CH_2OH), 2.95 (2 H, m, 6-$CH_2CH_2CONHNH_2$), 2.49 (2 H, m, 6-CH_2CH_2-$CONHNH_2$), and 1.90 (1 H, s).

Coupling of the Hydrazide (**4**) *to Carboxy-functionalised (CH–) Sepharose.*—Damp carboxy-functionalised (CH–) Sepharose (Pharmacia) was suspended in water (10 ml) and the hydrazide (**4**) (99.7 mg) in water (5 ml) was added. 1-Ethyl-3(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC. HCl) (0.35 g) was added to effect coupling. The pH was adjusted from 5.2 to 4.6 by addition of dilute hydrochloric acid, and the mixture was stirred gently for 24 h at room temperature. The product was collected on a sintered-glass funnel, and washed with 1M-disodium hydrogen phosphate (10 ml), water (10 ml), 1M-potassium dihydrogen phosphate (10 ml), and water, until the washings showed no absorbance at 350 nm. The 'bioadsorbent' was suspended in water (25 ml) and stored overnight at room temperature. The suspension was collected by filtration, and the Sepharose washed again until there was no further absorbance at 350 nm. The product was stored in 0.1M-potassium dihydrogen phosphate (pH 4.7) at 2 °C.

Synthesis of 6-(2-Hydrazinocarbonylethyl)-8-D-ribityl-pteridine-2,4,7(1H,3H,8H)-trione (**5**).—*6-(2-Methoxycarbonyl-ethyl)-8-D-ribitylpteridine-2,4,7(1H,3H,8H)-trione.* The 6-carboxyethylpteridine (3) (0.5 g, 1.2×10^{-3} mol) was placed in a dry flask and dry methanol (150 ml) was added. Concentrated sulphuric acid (5 drops) was added and the mixture was stirred under reflux in a water bath for 4 h. The resulting solution was set aside in the refrigerator overnight, and the white crystals were collected. The ester was recrystallised with difficulty from methanol, yielding *6-(2-methoxycarbonylethyl)-8-D-ribityl-pteridine-2,4,7(1H,3H,8H)-trione*, as white needles (0.42 g, 80%), m.p. 148—149 °C (Found: C, 44.6; H, 5.1; N, 13.0. $C_{15}H_{20}N_4O_9$ requires C, 45.0; H, 5.0; N, 14.0%); $\lambda_{max.}(\varepsilon)$ (pH 1) 282 (11 650) and 328 nm (11 680); $\lambda_{max.}$ (pH 13) 260 (8 130), 288 (7 770), and 355 nm (12 870); $\delta(D_2O-NaOD)$ 4.5—3.4 (7 H, m, ribityl envelope), 3.27 (3 H, s, CO_2CH_3), 2.89 (2 H, m, $CH_2CH_2CO_2CH_3$), and 2.55 (2 H, m, $CH_2CH_2CO_2CH_3$).

6-(2-Hydrazinocarbonylethyl)-8-D-ribitylpteridine-2,4,7-(1H,3H,8H)-trione (**5**). The 6-(2-methoxycarbonylethyl)-pteridine (1.25 g, 0.003 mol) was dissolved in hydrazine hydrate (20 ml) and stirred at room temperature for 1 h. Ethanol (150 ml) was added and the precipitate was collected, pressed, and washed thoroughly with ether. The cream coloured solid (0.75 g, 60%) was recrystallised with difficulty from 2M-acetic acid or 0.1M-hydrochloric acid to give *6-(2-hydrazinocarbonylethyl)-8-D-ribitylpteridine*-2,4,7(1H,3H,8H)-trione (**5**), m.p. 194—195 °C (Found: C, 41.8; H, 5.05; N, 20.9. $C_{14}H_{20}N_6O_8$ requires C, 42.0; H, 5.05; N, 21.0%); $\lambda_{max.}(\varepsilon)$ (pH 1) 283 (10 600) and 329 nm (10 600); $\lambda_{max.}$ (pH 13) 261 (9 070), 286 (6 900), and 357 nm (12 600); $\delta(D_2O-NaOD)$ 4.5—3.4 (7 H, m, ribityl envelope), 2.89 (2 H, m, CH_2CH_2CO), 2.55 (2 H, m, CH_2CH_2CO), and 1.90 (1 H, s).

Coupling of the Hydrazide (**5**) *to Carboxy-functionalised (CH–) Sepharose.*—Damp CH-Sepharose (2.5 g) was suspended in water (10 ml), and 6-(2-hydrazinocarbonylethyl)-8-D-ribityl-pteridine-2,4,7(1*H*,3*H*,8*H*)-trione (**5**) (100 mg) in water (5 ml) was added. 1-Ethyl-3-(3-dimethylaminopropyl)carbodi-imide hydrochloride (EDC.HCl) (0.35 g) was added to effect coupling. The suspension was adjusted to pH 4.6 and the mixture stirred gently for 24 h at room temperature. The product was collected on a sintered-glass funnel and washed with 1M-disodium hydrogen phosphate (10 ml), water (20 ml), 1M-potassium dihydrogen phosphate (10 ml), and water, until the washings showed no absorbance at 350 nm. The bioadsorbent was suspended in water (25 ml total) and stored overnight at room temperature. The suspension was collected and washed again till there was no further absorbance at 350 nm. The bioadsorbent was stored in 0.1M-potassium dihydrogen phosphate at 2 °C.

Initial Purification of Yeast Riboflavin Synthase.—1. *Extraction.* Dried baker's yeast (600 g) was stirred with de-ionised water (2 l) and toluene (38 ml) for 24—48 h at room temperature. The pH was then adjusted from 5.9 to 5.2 with dilute acetic acid, and the yeast cell debris removed by centrifugation (6 000 r.p.m., 20 min). The supernatant liquor (1 625 ml) was retained. Protein concentration = 14.4 mg/ml, *i.e.* 23.40 g protein in total. Total enzyme activity = 1 616 nmol riboflavin formed per min, *i.e.* a specific activity of 0.07 nmol riboflavin/mg/min at 37 °C.

2. *First ammonium sulphate precipitation.* The extract (1 615 ml) was stirred magnetically in a conical flask, and brought to 52% saturation by the addition of solid ammonium sulphate (525 g); stirring was continued for 1 h, and the suspension centrifuged (9 000 r.p.m., 30 min). The supernatant liquor was discarded and the residue was dissolved in 0.1M-ammonium acetate–0.01M-sodium sulphite solution (400 ml), giving a total volume of 430 ml. Protein concentration = 10.07 mg/ml, *i.e.* 4.329 g total protein. Total activity = 751 nmol riboflavin/min, *i.e.* a specific activity of 0.17 nmol riboflavin/mg/min at 37 °C.

3. *Second ammonium sulphate precipitation.* The ammonium acetate solution from step 2 (425 ml) was stirred in a conical flask and brought to 50% saturation by the addition of solid ammonium sulphate (118 g). Stirring was continued for 1 h, and the suspension centrifuged (9 000 r.p.m., 30 min). The supernatant liquor was discarded and the residue was taken up in 0.1M-potassium phosphate at pH 6.9 (200 ml), which was also 6×10^{-3}M in sodium hydrogen sulphite and 1×10^{-3}M in cysteine hydrochloride, giving a total of 208 ml. Protein concentration = 10.06 mg/ml, *i.e.* 2.115 g total protein. Total activity = 769 nmol riboflavin/min, *i.e.* a specific activity of 0.36 nmol riboflavin/mg/min at 37 °C.

4. *Dialysis.* The above potassium phosphate solution (205 ml) was dialysed against the same buffer (7 l) at 2 °C for 16 h, giving a total of 228 ml. Protein concentration = 9.17 mg/ml, *i.e.* 2.09 g total protein. Total activity = 946 nmol riboflavin/min, *i.e.* a

specific activity of 0.45 nmol riboflavin/mg/min at 37 °C. This dialysate was stored at 2 °C.

(5a). *Centrifugation.* When required for affinity chromatography, an aliquot of the dialysate was centrifuged (12 000 r.p.m., 30 min, 2 °C) and assayed afresh.

In some of the early affinity chromatography experiments (with materials A and B) an alternative purification procedure after the dialysis step (4 above) was followed.

(5b). *Acetone precipitation.* The solution from step 4 was made 0.2M in mercaptoethanol and adjusted to pH 8.3 by the addition of 8M-ammonium hydroxide. It was then cooled to 0 °C and brought to 48% v/v with acetone by the dropwise addition of acetone at −10 °C to the stirred enzyme solution. The mixture was stirred for 45 min at −10 °C and centrifuged at this temperature. The supernatant fluid was held at −10 °C and stirred while being brought to 70% v/v by the dropwise addition of acetone at −10 °C. After being stirred for 45 min at this temperature, the suspension was centrifuged while cold and the supernatant fluid was discarded. The residue was quickly dissolved at 0 °C in 0.04M-potassium phosphate buffer (100 ml) (pH 6.9) which was also 0.2M in mercaptoethanol. Total volume 103 ml. Protein 17.0 mg/ml. Total protein 1.75 g. Adsorbance at 470 nm of 0.16 was produced by 0.1 ml of this solution indicating an activity of 0.76 nmol/mg/min and a total activity of 1 327 nmol/min.* This solution was quickly frozen as a thin film with a solid CO_2–acetone bath and freeze-dried. The resulting solid material (1.39 g) was stored at −20 °C.

Affinity Chromatography Experiments.—All chromatographic procedures were carried out at 4 °C with automatic collection of 2.0 or 3.5 ml fractions, and continuous monitoring of the eluate for optical transmittance at 280 nm was carried out.

All assays of protein concentration were carried out using the method of Lowry *et al.*[16] with a standard of bovine serum albumin.

Assays of Riboflavin Synthase Activity.—When only low levels of enzymic activity or an excess of protein were present (*i.e.* when purifying the enzyme) the single point assay of Harvey and Plaut[14] was used. The enzyme was incubated with 10^{-4}M-6,7-dimethyl-8-D-ribityl-lumazine and 3×10^{-3}M-sodium hydrogen sulphite in 0.2M-potassium phosphate buffer (pH 6.9). At zero time and after 1 h an aliquot from the incubation mixture was quickly mixed with 0.5 volumes of ice-cold 15% trichloroacetic acid. Spectra from these sample pairs were obtained using a Pye-Unicam SP 8000 spectrophotometer, 10-mm path length, and optical density changes at 470 nm were used to calculate units of enzyme activity. Wavelength scans recorded with appropriate dilutions gave spectra which confirmed that the substrate lumazine was being converted into riboflavin.

Assays of the activity from the individual affinity columns varied and are described for each column.

Control Columns.—The column was packed under gravity with Sepharose 4B (approx. 5 ml) and was well washed before use with 0.2M-potassium phosphate buffer (pH 6.9). The enzyme was purified with acetone before being freeze-dried (5.0 ml) and was then loaded onto the column and eluted with sufficient buffer to remove all unadsorbed protein, as measured by the transmittance at 280 nm. The protein peak passed straight through the column with no retardation. Assays of protein-containing fractions for riboflavin synthase activity were carried out on solutions containing: (a) 0.4 ml of protein fraction; (b) 0.2 ml of 6×10^{-4}M-lumazine (1) in 0.2M-phosphate buffer (pH 6.9); (c) 0.2 ml of 6×10^{-3} M-sodium hydrogen sulphite in 0.2M-phosphate buffer (pH 6.9); and (d) 0.4 ml of 0.2M-phosphate buffer (pH 6.9) equilibrated at 37 °C in a semimicro cuvette of 10-mm pathlength.

The initial velocity of the reaction was recorded during 10 min by measuring the increase in absorbance at 470 nm using a recording spectrophotometer with linear readout. Specific activities of the enzyme were calculated as described previously and are expressed as nmol riboflavin formed/mg protein/min.

Using this assay it was established that the riboflavin synthase activity was located in the centre of the protein peak and recovery was essentially quantitative.

A CH-Sepharose column run under identical conditions gave similar results with 85% recovery of enzyme activity.

A column of AH-sepharose slightly retarded activity so that the maximum enzyme activity was detected after most of the protein had left the column. The recovery of activity was 90%.

Affinity Chromatography using Material A (see Figure).—Material A (approx. 5 ml) was packed and washed as in the control columns described above. Acetone purified enzyme (2 ml + 2 ml + 1 ml) was loaded in three separate batches (at points *a* in Figure) and the column was eluted with 0.2M-potassium phosphate buffer (pH 6.9). The unadsorbed protein peaks in the eluant were identified from the transmittance at 280 nm, and these fractions were assayed for enzyme activity using the procedure described for the control columns. No activity breakthrough was observed. Substrate elution (50 ml; 5×10^{-3}M) in 0.2M-potassium phosphate buffer (pH 6.9 containing 10^{-3}M-cysteine hydrochloride) was started at point *b* (see Figure). All of the yellow substrate-containing fractions were assayed for riboflavin synthase activity on solutions prepared as follows: (a) 1.5 ml of eluant; (b) 0.5 ml of 1.8×10^{-2}M-sodium hydrogen sulphite in the phosphate buffer (pH 6.9); and (c) 1.0 ml of the phosphate buffer (pH 6.9.). These were equilibrated to 37 °C and then assayed as described above.

From this the activity elution profile shown in the Figure was obtained. Calculation of the total eluted activity showed that quantitative recovery had been achieved. A similar experiment in which the enzyme was loaded in one batch led to 7% leakage of enzyme and 81% recovery.

Estimation of the Purification Factor using Material A.—Crude enzyme from the dialysis step 4 (5.0 ml; specific activity 1.35 nmol riboflavin/min/mg protein) was loaded on to the column (approx. 5 ml of material A) which was then washed with 0.02M-potassium phosphate buffer (pH 7.0) (5.0 ml). The column was then eluted with lumazine (1) (4.5×10^{-3}M) in 0.02M-potassium phosphate buffer (pH 7.0) (15 ml) and the fractions (approx. 3 ml) were assayed directly for enzyme activity after equilibration at 37 °C. Activity was observed in the substrate-containing fractions 2—4 and these were combined (total volume 9.25 ml). The activity of this combined material was 2.6 nmol riboflavin/min/ml.

This yellow solution was dialysed against 2×10^{-3}M-potassium phosphate buffer (3 000 ml changed twice) at 0 °C. After 3 days of dialysis the solution inside the bag was colourless and showed no lumazine chromophore. This colourless material (14.4 ml), assayed as described above, showed no activity. The protein concentration of this solution was 0.03 mg/ml which enabled a specific activity to be calculated for the eluted material (56 nmol riboflavin/min/mg protein) which represents a 42-fold purification of the enzyme.

Affinity Chromatography using Material B.—The experimental procedure described for material A was repeated using material B with the following differences. Acetone-purified

* These activity values were obtained from material isolated in steps 1—4 in a separate but identical experiment and therefore are representative ***only*** **of this final step (5b).**

enzyme (7.8 ml) was applied to this column in one batch and approx. 10% breakthrough of activity occurred. Substrate elution (5×10^{-3}M) gave a very broad peak of low activity; however, when the substrate concentration was increased to 10^{-2}M the activity became sharp. Approximately 15% activity recovery was achieved at 5×10^{-3}M-substrate whereas 49% recovery was achieved at 10^{-2}M-substrate.

Affinity Chromatography using Material C.—Experiments on material C were run at a later date and were under conditions modified from the earlier experiments.

Substrate elution. Material C (5 ml) was packed in a column and equilibrated with 'washing buffer' (20 ml) (0.1M-potassium phosphate, pH 6.9, containing 6×10^{-3}M-sodium hydrogen sulphite and 10^{-3}M-cysteine hydrochloride). Riboflavin synthase activity (5 ml) from the dialysis step 4 was applied to the column which was then eluted with 'washing buffer' (30 ml) until the transmittance at 280 nm returned to its original base line. Protein-containing fractions were then assayed for activity using the following conditions: 'washing buffer' (2×4 ml) and 4.5×10^{-3}M-substrate (**1**) (1.0 ml) were equilibrated at 37 °C for 10 min; then the eluted protein fraction (0.5 ml) was added and equilibration continued for a further 5 min, after which time the rate of increase in absorbance at 470 nm was measured. These protein-containing fractions showed no activity.

The affinity column was then eluted with 10^{-2}M-substrate (**1**) in 'washing buffer' (40 ml). The fractions containing the yellow substrates were assayed for activity in the following way: 'washing buffer' (2.5 ml) was equilibrated at 37 °C for 10 min. The yellow eluted fraction (0.5 ml) was added and equilibration continued for a further 5 min, after which time the rate of increase in absorbance at 470 nm was measured. A recovery of activity of 78% of that applied to the column was achieved in six fractions (3 ml) with a sharp elution profile.

A repeat of this experiment under identical conditions again gave no breakthrough and 95% of the applied activity was recovered; however, the elution profile in this experiment was broader than the above.

Removal of Substrate.—The yellow fractions were immediately applied to Sephadex G10 (35×3.5 cm) and eluted with degassed water. The protein was eluted immediately following one column volume and contained 50% of the applied enzyme activity.

Stability and Purity of the Enzyme.—The stability of the purified enzyme in washing buffer in air is very low. At room temperature or at 2 °C complete loss of activity occurs in 20 h. At −80 °C, 10% of the activity is lost after 21 h and 25% after 72 h. Enzyme which had been freeze-dried lost 60% of its activity after being stored for 72 h at −20 °C.

Analytical gel electrophoresis was carried out on a freeze-dried sample of riboflavin synthase. Samples were tested at pH 6.6 and 8.5 on polyacrylamide gels, polymerised using riboflavin as the photoinitiator, and at pH 6.6 on gels which used ammonium persulphate as initiator. No protein bands were observed with the riboflavin-initiated gels, whereas large areas of the gel seemed to be stained with the latter initiator. One early experiment with the latter gel gave one clearly stained protein band but this has not been reproducible. No conclusion regarding enzyme purity can therefore be reached from these attempts.

Estimation of the Purification Factor using Material C.—Using the chromatography procedure described above (material C) and the indirect method for the calculation of the specific activity of purified enzyme, riboflavin synthase having a specific activity of 0.48 nmol riboflavin/min/mg protein was purified by a factor of 86 times to a final specific activity of 41.5 nmol riboflavin/min/mg protein.

Another experiment using crude material of the same specific activity achieved a purification factor of 134 times to give a final specific activity of 64 nmol riboflavin/min/mg protein.

Acknowledgements

We thank the S.E.R.C. and Burroughs Wellcome for financial support.

References

1 Part 4, S. S. Al-Hassan, I. Stirling, and H. C. S. Wood, *J. Chem. Res.*, 1980, (*S*) 278; (*M*) 3630.
2 S. S. Al-Hassan, R. J. Kulick, D. B. Livingstone, H. C. S. Wood, R. Wrigglesworth, and R. Ferone, *J. Chem. Soc., Perkin Trans. 1*, 1980, 2645.
3 T. Paterson and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1972, 1041.
4 H. C. S. Wood, R. Wrigglesworth, D. A. Yeowell, F. W. Gurney, and B. S. Hurlbert, *J. Chem. Soc., Perkin Trans. 1*, 1974, 1225.
5 T. Paterson and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1972, 1051.
6 R. J. Kulick, H. C. S. Wood, R. Wrigglesworth, and C. D. Ginger, *J. Chem. Soc., Chem. Commun.*, 1975, 464; R. Wrigglesworth, C. D. Ginger, R. J. Kulick, and H. C. S. Wood, in 'Chemistry and Biology of Pteridines,' ed. W. Pfleiderer, de Gruyter, Berlin, 1978, p. 93.
7 P. Cuatrecasas, *J. Biol. Chem.*, 1970, **245**, 3059; E. Steers, P. Cuatrecasas, and H. B. Pollard, *ibid.*, 1971, **246**, 196.
8 T. Lang, C. J. Suckling, and H. C. S. Wood, *J. Chem. Soc., Perkin Trans. 1*, 1977, 2189.
9 G. W. E. Plaut, *J. Biol. Chem.*, 1963, **238**, 2225.
10 C. R. Lowe and P. D. G. Dean, 'Affinity Chromatography,' Wiley, London, 1974, ch. 2.
11 'Methods of Enzymatic Analysis,' ed. H. U. Bergmeyer, Verlag-Chemie, Weinheim, 1974, vol. 1, p. 522.
12 E. C. Slater and W. B. Banner, *Biochem. J.*, 1952, **52**, 155.
13 'Methods of Enzymatic Analysis,' ed. H. U. Bergmeyer, Verlag-Chemie, Weinheim, 1974, vol. 4, p. 2246.
14 R. A. Harvey and G. W. E. Plaut, *J. Biol. Chem.*, 1966, **241**, 2120.
15 R. M. Cresswell and H. C. S. Wood, *J. Chem. Soc.*, 1960, 4768.
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1951, **193**, 265.

Received 21st September 1983; *Paper* 3/1660